IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Gregory S. HAMILTON *et al.*

Appl. No. 09/994,927

Filed: November 28, 2001

For: **Bisubstituted Carbocyclic Cyclophilin Binding Compounds and their Use**

Art Unit: (unassigned)

Examiner: (unassigned)

Atty. Docket: 03166.0029.NPUS00

Marked Up Version to Show Changes Made

Commissioner for Patents
Washington, D.C. 20231

Sir:

Please amend the application as follows:

In the Specification:

Please insert the accompanying Sequence Listing, containing 1 page and SEQ ID NOs. 1-3.

Please replace the three paragraphs beginning on page 97, line 10 of the specification and ending on page 98, line 15, with the following replacement paragraphs:

> A number of substrates for rotamase are known in the art or can be derived from those known. Typically, the substrate contacts a sample containing a protein with rotamase activity and the conversion of the substrate is detected after a period of time. The method for detecting conversion of the substrate will vary with the particular substrate chosen. One method has been termed the Ki test (See Harding, et al., Nature, 341:758-760 (1989). The cis-trans isomerization of an alanine-proline bond in a model substrate, N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (SEQ ID NO: 1), is monitored spectrophotometrically in a chymotrypsin-coupled assay. The action of chymotrypsin releases p-nitroaniline from only the trans form of the substrate. The amount of p-nitroaniline can be

monitored in a spectrophotometer, for example. Other methods of detecting the presence of p-nitroaniline can also be used. The inhibition of this reaction caused by different concentrations of inhibitor is determined and the data are analyzed as a change in first-order rate constant as a function of inhibitor concentration, which yields the Ki value.

The following were added to a plastic cuvette: 950 mL of ice cold assay buffer (25 mM HEPES, pH 7.8, 100 mM NaCl), 10 μL of CyP A (2.5 μM in 10 mM Tris-Cl pH 7.5, 100 mM NaCl, 1 mM dithiothreitol), 25 μL of chymotrypsin (50 mg/ml in 1 mM HCl), and 10 μL of test compound, at various concentrations, in dimethyl sulfoxide. The reaction was initiated by the addition of 5 μL of substrate (succinyl-Ala-Phe-Pro-Phe-para-nitroanilide (SEQ ID NO: 1), 5 mg/mL in 470 mM LiCl in trifluoroethanol). The absorbance at 390 nm versus time was monitored for 90 seconds using a spectrophotometer and the rate constants were determined from the absorbance versus time data files.

The IC50 values that were obtained for representative compounds in the following Table I refer to the concentration that inhibits 50% of the rotamase activity in a sample. The lower the value, the more active the compound is at binding or interacting with CyP. The Cyclophilin utilized was a recombinant rat CyPA-GST fusion protein: CypA was amplified from rat brain cDNA using standard PCR methods, primed with the following sequences: 5' CCC CCC GGG AGT CAA CCC CAC CGT GTT CTT CGA 3' (SEQ ID NO: 2) and 5' GGA GAT CTA GAG TTG TCC ACA GTC GGA GAT GGT 3' (SEQ ID NO: 3). The resulting fragment (573 base pairs) was cloned into pCRII and amplified. The CyP sequence was cut out with SmaI and EcoR1 and cloned into the SmaI/EcoR1 sites in pGEX2TK (Pharmacia). This plasmid was transformed into BL21 E. coli cells for expression of the GST-CyPA fusion protein. An asterisk indicates that the compound was evaluated using a human recombinant CyPA, [Yoo et al., J. Mol. Biol., 269 (1997) 780-95].